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Circulating Cytokines Response and the Level of Erythropoiesis in Sickle Cell Anemia

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A hemoglobin F (HbF) level between eight and nine percent divides sickle cell anemia (SS) patients into two populations, according to the kinetics of circulating burst forming units-erythroid (BFU-E), long term culture-initialing cells (LTC-IC), and cytokine plasma concentrations. The SS patients with HbF levels lower than 8–9% are more anemic (LFSS patients) than those with HbF levels higher than 8–9% who have less severe anemia (HFSS patients).

We report here that the level of erythropoiesis [evaluated by the levels of soluble transferin receptors (sTfR)] is not identical in these two patient populations, supporting the idea that a different set of regulatory mechanisms might be required to maintain the two levels of increased hematopoiesis. The plasma sTfR concentration was increased in all SS samples compared with controls ($P < 0.002$) and sTfR levels were negatively correlated with peripheral HbF%. ($r = -0.574$, $P < 0.002$). Furthermore, sTfR levels were higher in LFSS than in HFSS patients. Erythropoietin (Epo) levels were increased in the plasma of LFSS individuals (range = 34–215 ml U/ml), while the values in HFSS patients were in the normal range (3–20 ml U/ml).

Furthermore, we identify here stem cell factor (SCF) and transforming growth factor-β (TGF-β) as regulatory factors specifically affected by the presence of SS genotype and its level of severity. The plasma concentrations of SCF and TGF-β were increased compared with normal controls and high levels of SCF (up to 7,000 pg/ml) were detected in LFSS patients. The latter also showed increased proportion of SCF^{+} CD^{34} enriched circulating cells (49%). Low SCF in HFSS patients is associated with elevated TGF-β, suggesting a regulatory role of the latter on either SCF release or c-kit expression in progenitor cells.

Occasional elevation of granulocyte macrophage-colony stimulating factor (G-CSF), interleukin (IL)-7, and macrophage inflammatory protein (MIP)-1α in plasma of SS patients is not specific because no relation to HbF could be demonstrated. All plasma tested for leukemia inhibitory factor (LIF) were negative.

Data presented here, complementing previously published information, supports a model in which HFSS patients achieve a balance between inhibitory (TGF-β) and stimulatory (SCF, IL-3) factors, resulting in moderate erythropoietic response. In contrast, in LFSS patients, low levels of TGF-β and the increased release of GM-CSF and SCF maintain the intense erythropoiesis in response to higher erythropoietic stress, in these more severe patients. Am. J. Hematol. 60:105–115, 1999.

Key words: steel factor; TGF-β; transferrin receptor; fetal hemoglobin; hematopoiesis

INTRODUCTION

Hematopoiesis in under the control of competing cytokines: some directing differentiation, some enhancing proliferation, and some inhibiting these processes. In response to stress or pathological conditions, cytokines are released and the proliferation and/or differentiation of stem cells are modified.

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We have previously shown that sickle cell anemia (SS) is associated with endogenous production of growth factors [1–3] which could lead to various degrees of activation, mobilization, and differentiation of hematopoietic stem cells [4]. These effects are different in the group of SS patients with low hemoglobin (Hb)F (<8–9% HbF, LFSS patients) characterized by significant anemia, compared with patients with a less severe form of the disease associated with high HbF (>8–9% HbF, HFSS patients) [5]. There is no doubt that HbF inhibits polymerization of HbS in red cells in vivo [6] as well as in vitro and thus has a mitigating effect on the pathophysiology of the disease.

The question remains, are the LFSS and HFSS patients different in the level of erythropoietic response? To answer this question we have measured the levels of soluble transferin receptors (sTfR) in the plasma of SS patients.

The levels of sTfR in plasma or serum are an indirect measure of total TfR [7–10] and because most TfR is confined to the erythroid progenitor cells [10–12], the plasma/serum levels of sTfR reflect the size of the erythroid progenitors' pool or the rate of erythropoiesis.

We have previously reported that granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and/or, as yet, an unidentified inhibitor [1–3] are involved in the regulation of hematopoiesis in SS disease, and that they are expressed differently in SS patients with high and low HbF. Nevertheless, no data exist on stem cell factor (SCF), transforming growth factor (TGF)-β, granulocyte stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1α, leukemia inhibitory factor (LIF), and interleukin-7 (IL)-7 in SS anemia. This work will report on these factors' role in the erythropoiesis of SS disease and propose a model for their interaction.

SCF, the c-kit ligand (also called ''a mast factor''), has been implicated in many physiologic processes including melanocyte migration, primordial germ-cell development, mast cell function, and hematopoiesis [13–16]. SCF acts at an early differentiation level (similar to Flt ligand) and therefore has the capacity to stimulate the earliest hematopoietic stem cells. SCF, in a synergy with other hematopoietic growth factors in vitro, promotes growth of stem cells [17–21] and stimulates colony formation by precursor cells from patients with hypoproliferative disorders: Diamond-Blackfan anemia [22–24] and aplastic anemia [25–27]. Furthermore, SCF/c-kit interaction is required during acute expansion of hematopoietic cells in murine spleen [28]. Taken together, these results suggested that SCF may be involved in the regulation of stress hematopoiesis in SS anemia. To test this hypothesis, we evaluated the levels of SCF in plasma of SS patients with high and low HbF levels; we also determined the proportion of SCF^{+} cells in the enriched circulating CD^{34+} population in the same individuals.

TGF-β is a member of the cytokine family whose major effects are on differentiation and the inhibition of cell growth. There is evidence that TGF-β reversibly inhibits cell proliferation by extending or arresting the G_1 phase of the cell cycle [29]. TGF-β is a potent negative regulator of hematopoiesis in long-term bone-marrow (LTBM) cultures [30,31]. Whereas TGF-β functions predominantly as a negative regulator of cell growth, it may also exhibit a stimulatory effect as demonstrated by GM-CSF–dependent stem cell proliferation [32]. On the basis of these observations, we hypothesized that TGF-β may be a bifunctional modulator of the hematopoiesis in SS anemia, and thus its plasma concentrations will be different in LFSS and HFSS individuals and related to HbF and SCF levels.

G-CSF, IL-7, LIF, and MIP-1α factors, whose production and release may be modified in various pathological conditions, were also evaluated. G-CSF is a lineage specific hematopoietic growth factor for neutrophils [33,34]. G-CSF can be detected in patients with bacterial or fungal infections in the range between 76 and 370 pg/ml; this elevation is transitory and returns to the normal level as the patient recovers. Increased levels of G-CSF up to 2,000 pg/ml has been also reported in various hematological disorders [35,36].

Darnia et al. [37] reported that IL-7 differentially regulates myelopoiesis in the mouse bone marrow and spleen, while stimulating lymphopoiesis and orchestrating the redistribution of stem cells. Thus, IL-7 may be implicated in the increased mobilization of SS stem cells.

LIF is a multifunctional cytokine, which shares many bioactivities with IL-6, including regulation of the differentiation and proliferation of certain hematopoietic cell lines [38]. Additionally, LIF possesses unique functions, including inhibition of embryonic stem cell differentiation [39].

MIP-1α has been described as a potent inhibitor of hematopoietic stem cell growth [40,41]. We postulated that LIF and/or MIP-1α are other candidates involved in negative regulation of sickle cell anemia stem cells, especially in HFSS patients.

MATERIALS AND METHODS

Patients

We studied 53 patients homozygous for HbS (SS) in steady state (no acute complications). Their ages ranged from 28 to 45. Their average laboratory values were: Hb 7.82 ± 1.78 g/dl; HbF ranged from 0.9 to 25%; total bilirubin 3.4 ± 3.5 mg/dl; indirect bilirubin 0.67 ± 0.4 mg/dl; SS dense cells 15.1 ± 11.6; SS platelets 417 ± 41.9 $\times 10^3/\mu l$; LFSS platelets 436 ± 54.8 $\times 10^3/\mu l$; and HFSS platelets 395 ± 67.4 $\times 10^3/\mu l$. (The number of platelets in

LFSS patients is not significantly different from HFSS individuals $P = 0.32$). Informed consent was obtained before venepuncture when the samples were not part of the patient's normal medical care. Ten normal volunteers were used as controls.

Cytokine Evaluation

Using a commercially available enzyme-linked immunoabsorbent assay (Predicta Genzyme, R&D Systems, Minneapolis, MN), cytokine plasma levels were determined in patients with HbF levels ranging from 0.9–25% and 10 controls. Plasma was harvested, and aliquots were made and kept at −60°C until use. Cytokines were quantitated by an Elisa solid enzyme immunoassay employing the multiple-sandwich principle. Bound immunoreactive cytokine is quantitated by enzymatic reaction resulting in a color change detectable by an Elisa reader. The results were calculated as recommended by the manufacturer.

Enrichment of CD^{34+} Cells

Mononuclear cells were separated from 50 ml of heparinized peripheral blood by 30 min of centrifugation at 400*g* in Ficoll-Hypaque (density, 1,077 g/ml; Pharmacia Fine Chemicals AB, Uppsala, Sweden) (LD cells). The cells from the interface were washed twice with alpha medium supplemented with 2% heat-inactivation fetal calf serum (FCS) and enumerated. LD cells were resuspended in 4 ml of Dulbecco's phosphate buffered salt solution 1× without calcium and magnesium (DPBS/CMF) containing 0.5% human gamma globulin and incubated for 15 min at room temperature. Two step CD^{34+} cells separation was performed using a Micro CELLector Cell Culture Flask (Applied Immune Sciences [AIS], Inc.). In the first step the LD cell suspension was loaded into T-25 soybean agglutinin (SBA) flask (up to a total number of 6×10^7 cells per flask). Cells possessing surface N-acetyl galactosamine or its derivatives bind to the SBA-coated surface. Such cells include human T and B cells, monocytes, and red blood cells. After 1 hr of incubation at room temperature, the nonadherent SBA^- cells, enriched with CD^{34+} cells by negative selection, were removed and introduced into the CD^{34} AIS device covalently coupled with anti-CD^{34} (ICH3) antibody, for positive selection of CD^{34+} cells. At the end of 1 hr of incubation, the nonadherent CD^{34-} cells were removed and 4 ml of D-PBS/CMF containing 10% FCS was added to the flask to dislodge and recover adherent CD^{34+} cells. (As demonstrated by fluorescence-activated cell sorter [FACS] analysis, the average recovery of CD^{34+} cells was 80%; data not shown.) The CD^{34+} cells were enumerated and stained with biotinylated SCF avidin fluorescein isothiocyanate (FITC) as recommended by the manufacturer and FACS analysis was performed. Biotinylated SCF was purchased from R&D systems (Minneapolis, MN).

Flow Cytometry

FACS multiparameter analysis was done using a Becton Dickinson FACsort flow cytometer equipped with a 488-nm single air-cooled laser. The instrument was calibrated using the calibrate beads (Becton Dickinson) and fluorescence emission was collected through a 530/30 band pass filter for FITC. Unstained cells were used to adjust the baseline for the negative population. In most experiments, 10,000 events were acquired and analysis was done in gated lymphocyte windows selected by dot plots and presented as fluorescence distribution in contour plots using the Lysis II software (Becton Dickinson).

HbF Determination

A 1-min alkali denaturation test was used to determine the percentage of peripheral HbF in hemolysates prepared from SS patients' red cells as described [42]. Normal controls had HbF levels of less than 1%.

The statistical analyses were performed using the Statgraphics Plus program (SAS System Statistical Graphics Corporation, Rockville, MD).

RESULTS

sTfR Levels in LFSS and HFSS Patients

The levels of sTfR were determined in 26 plasma samples from SS patients with the HbF ranging from 0.88–15.2% and controls. The sTfR values in all SS plasma were significantly higher ($P < 0.002$) than those detected in controls (Black and Caucasian AA individuals): 73.4 ± 1.8 vs. 41 ± 7.9 ng/ml. Also, LFSS patients exhibited significantly higher ($P < 0.027$) levels of sTfR (76.6 ± 1.2 ng/ml) than HFSS individuals (67 ± 3.5 ng/ml) and regression analysis of the data revealed that the sTfR concentration was negatively correlated with peripheral level of HbF ($r = -0.5744$, $P < 0.002$) of each individual (Fig. 1).

Epo Levels in LFSS and HFSS Patients

The patients evaluated in this study had plasma erythropoietin (Epo) concentrations in the range from 20–215 ml/ml; mean value in controls was 5.27 ± 0.73 ml/ml. Although we did not find a significant correlation between Epo levels and peripheral HbF concentrations, we did find that the LFSS individuals tended to have statistically higher levels of Epo (90.7 ± 15 ml/ml) than their HFSS counterparts (16.2 ± 3.7 ml of Epo/ml) ($P < 0.001$). The HFSS Epo levels were not significantly different from controls ($P < 0.1065$).

SCF Levels in LFSS and HFSS Patients

The levels of SCF were determined in the 53 plasma samples from 21 SS patients with HbF ranging from 0.9

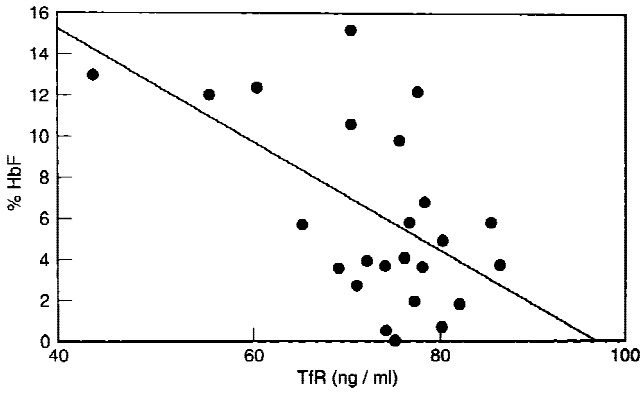

Fig. 1. The sTfR plasma levels were determined in 26 SS patients (20 LFSS and 6 HFSS individuals) and the values were correlated with peripheral HbF levels of each individual (on two occasions the values were the same and are not represented as double dots on the plot). The Y axis of the Figure represents the % of HbF, the X axis represents the TfR plasma concentrations. As shown, regression analysis of the data demonstrated that sTfR concentration was negatively correlated with peripheral HbF in each patient. (r = 0.5744, *P* < 0.021).

to 23% and ten controls. The results (Fig. 2) showed that SCF concentrations varied greatly between SS patients when compared with controls, 0–7,000 pg/ml vs. 1,200 pg/ml to 2,050 pg/ml in AA; these are values similar to those indicated by the manufacturer. However, when comparing LFSS and HFSS individuals, we found that LFSS had normal or elevated levels of SCF (m = 1938 ± 1168 pg/ml), whereas in HFSS patients, SCF tended to be below normal (m = 1209 ± 550 pg/ml).

The Box and Whisker statistical analysis showed that the difference between SCF levels in HFSS and LFSS patients was highly significant (using the comparison of the means procedure and assuming unequal variances, the *P* value was <0.002, and when the F test procedure to compare standard deviations was applied, the *P* value was <0.0002). The Kolmogorov-Smirnov test also demonstrated statistically significant differences between the SCF distributions in LFSS and HFSS patients ($P < 0.0139$). Regression analysis of the data further demonstrated that SCF was negatively correlated with the level of HbF ($r = -0.5706$, $P < 0.0069$). These results suggest that the SCF release is related to the rate of erythropoiesis and may be implicated in the erythroid expansion in which pre-CFC (early hematopoietic stem cells) are actively involved [43].

The samples from five LFSS and five HFSS patients were collected on 2–4 occasions over a 1–2-year period and the levels of SCF were determined. The data showed (Fig. 3A) that in 2/5 LFSS individuals, the level of SCF remained stable during follow-up, while in 3/5 LFSS patients, SCF concentration varied, sometimes dramatically. In contrast, the HFSS SCF plasma concentrations showed normal or below normal values during the study (Fig. 3B). While three out of five patients remained stable during follow up, the other two showed variations within the low concentration range of SCF.

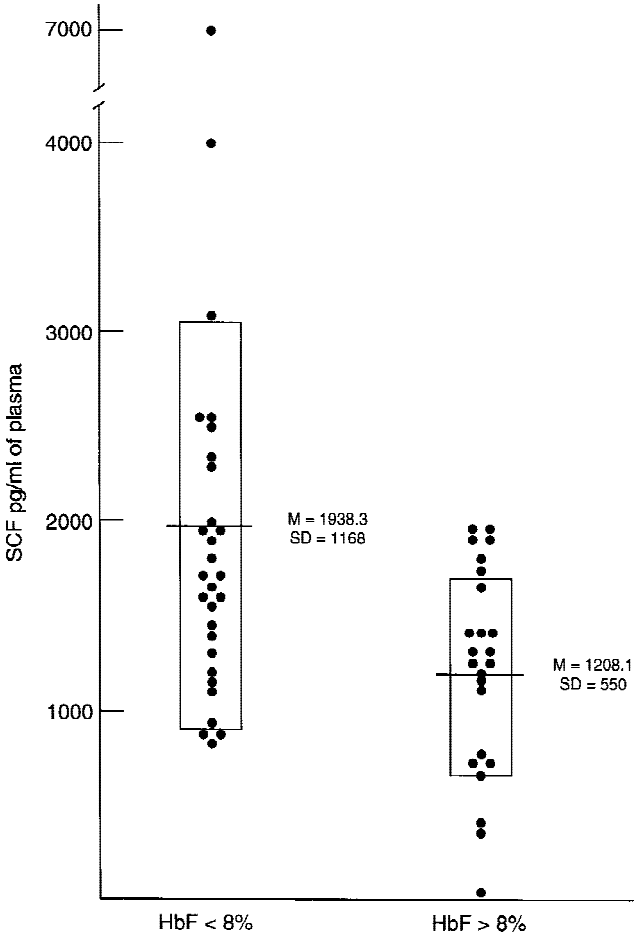

Fig. 2. The SCF levels were determined in 29 LFSS (HbF < 8.5%) (m = 1,938 pg/ml) and 23 HFSS samples (>8.5%) (m = 1,209 pg/ml). The Y axis of the Figure represents SCF concentration and the X axis represents the % HbF; dots represent individual values. The plot represents Box and Whisker statistical analysis designed to compare two samples of the data. The *t*-test analysis demonstrated that the difference in SCF levels between LFSS and HFSS patients is statistically different *P* < 0.002 (assuming not equal variances) and the Kolmogorov-Smirnov test further confirmed this conclusion (*P* < 0.0131).

Analysis of SCF Binding by Enriched CD^{34+} SS Cells

Representative FACS analysis of circulating SCF^{+} cells from an LFSS (HbF = 3.9%) patient, an HFSS (HbF = 22%) patient, and an AA individual are presented in Figure 4. Panels A, C, and E show contour plots of unstained cells from the HFSS, LFSS, and AA indi-

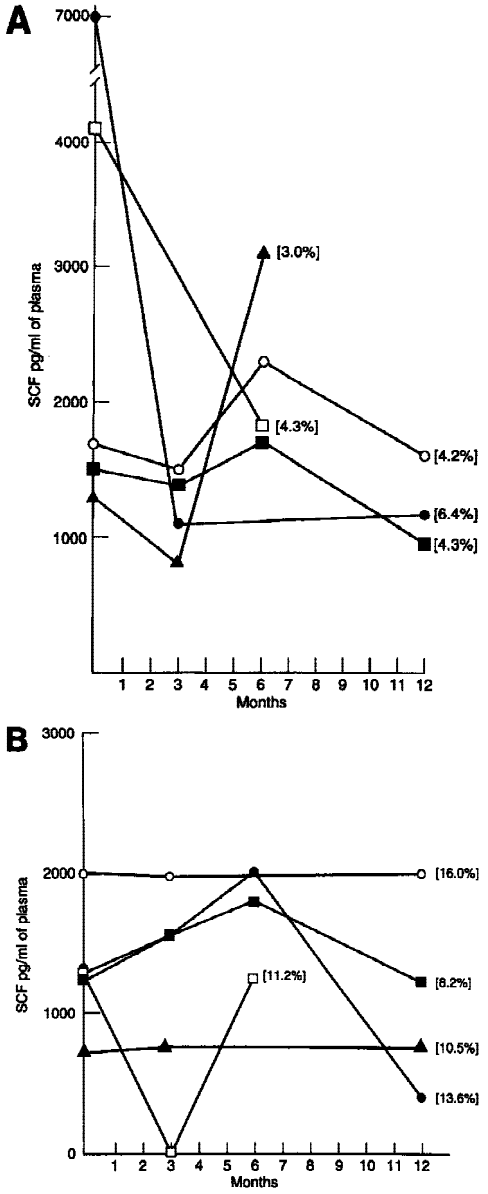

Fig. 3. A: Time course of SCF levels in plasma of five LFSS patients with HbF ranging from 3.1 to 6.4%. Solid lines denote the SCF concentrations evaluated over a 6 to 12 month period in each individual, and the number in the brackets indicates the percentage of peripheral HbF. Different symbols are used for each patient. The data show that in 2/5 patients the levels of SCF remain stable during follow-up, while in 3/5, the SCF concentration varies. B: Time course evaluation of SCF levels in plasma of five patients with HbF ranging from 8.2 to 16%. Different symbols are used for each patient and are connected by solid lines. The data show that SCF levels in HFSS patients are normal or below normal during the follow-up.

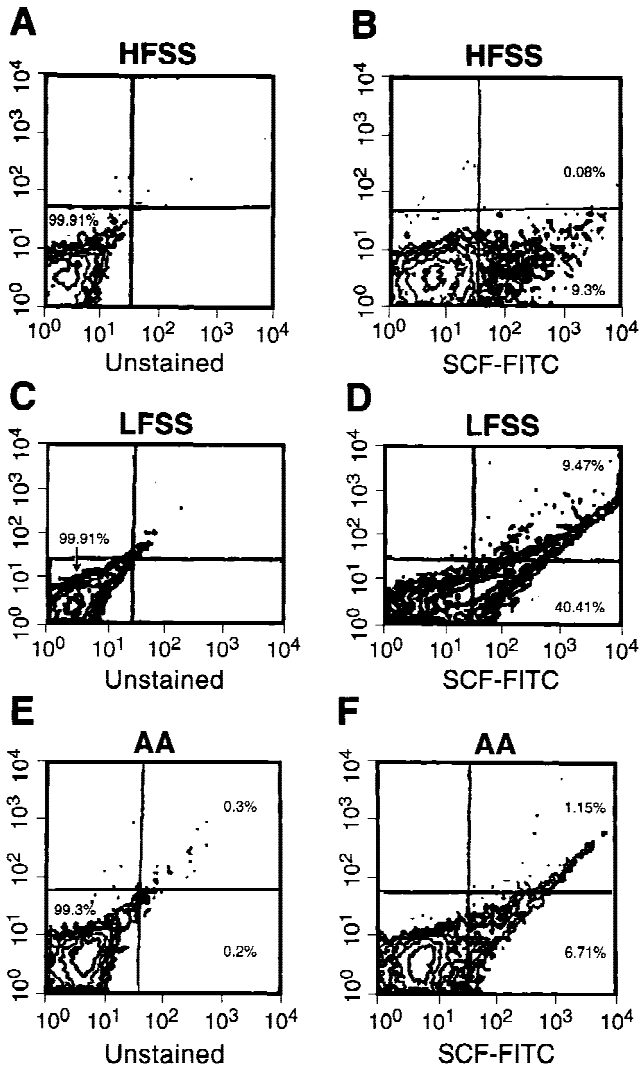

Fig. 4. Contour plot analysis of CD^{34} enriched cells stained with biotinylated SCF from one HFSS, one LFSS patient, and a control. Panels A, C, and E represent unstained CD^{34} enriched populations (panel A = HFSS; panel C = LFSS; panel E = control). Panel B shows that only 9.3% of HFSS CD^{34} enriched cells bind SCF as compared with 49% in the LFSS population (panel D). Panel F illustrates that 7.8% of the AA CD^{34} enriched population bind SCF.

viduals, used to adjust the baseline for the negative population. Figure 4, panel B shows that only 9.3% of HFSS circulating CD^{34+} cells bind SCF, whereas as much as 49.8% of LFSS CD^{34+} cells bind SCF (panel D). The AA individual (panel E) exhibits the similar proportion of circulating SCF^+ cells (7.8%) as HFSS patients. The labeling experiments were performed in six SS and three AA samples.

The proportion of CD^{34+} enriched SCF^+ cells from each SS individual were then correlated with the HbF level of the same person. The regression analysis demonstrated (Fig. 5A) that a highly significant negative correlation between the SCF^+ cells and the peripheral HbF level exists ($r = -0.937$; $P < 0.0188$). The individual SCF plasma concentrations were also correlated with the numbers of CD^{34+} SCF^+ cells detected in the same indi-

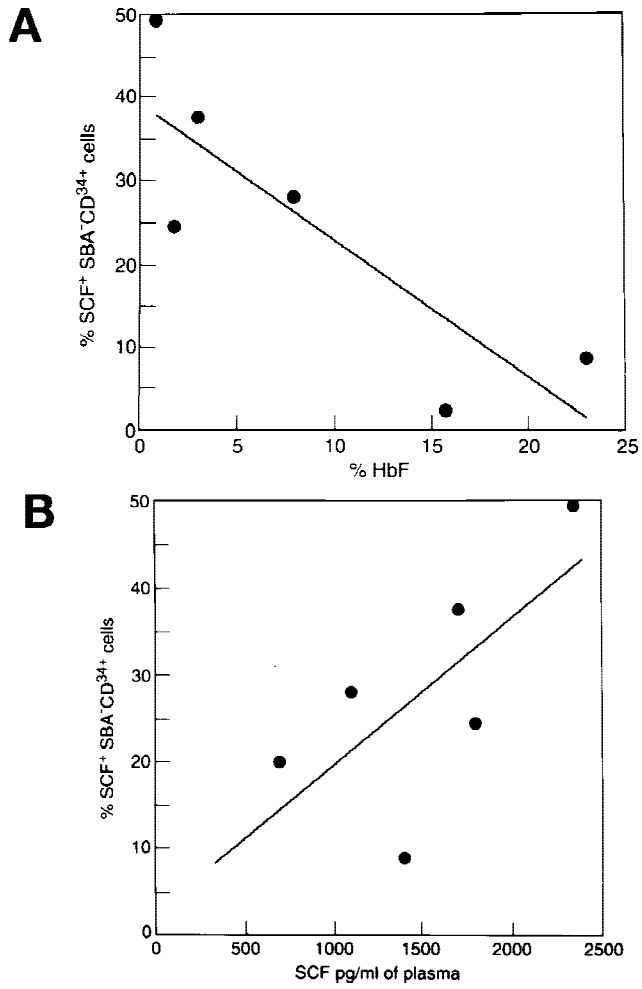

Fig. 5. A: The number of CD^{34+} SCF^{+} cells was determined in six SS patients using Fax analysis and correlated with the HbF levels of the same individual. The results show strong negative correlation between CD^{34+} SCF^{+} cells and peripheral HbF in SS patients ® = −0.937; $P < 0.0188$). B: The number of CD^{34+} SCF^{+} cells was also correlated with the SCF plasma concentration of the same individual. The regression analysis shows absence of correlation in the given samples. ($r = 0.6765$; $r^2 = 0.4576$; $p = 0.1400$).

vidual; regressioni analysis (Fig. 5B) showed absence of significant correlation between these two values ($r = 0.6765$, $r^2 = 0.4576$; $P < 0.14$). When the outlayer was removed and the regression analysis repeated, the r value was 0.81, the $r^2 = 0.65$, and the $P < 0.099$.

G-CSF and IL-7 Levels in LFSS and HFSS Patients

Twenty-one SS patient plasmas were evaluated for G-CSF. Sixteen had levels of G-CSF that fell below the lowest point of standard curve; five patients were positive and showed a range between 156 pg/ml and 312 pg/ml of G-CSF; however, no correlation between G-CSF concentration and HbF levels could be demonstrated. Normal African-American donors were negative. These results suggest that an occasional increase of G-CSF is not specific for the pathophysiology of SS disease and probably reflects a nonspecific increase of G-CSF in response to infection or inflammation, events that are very common in sickle cell disease.

Thirty-two plasmas from SS patients were evaluated for IL-7. The level of IL-7 in 30 patients was below 10 pg/ml; in two patients the IL-7 level was 14.0 and 12.5 pg/ml, respectively. The normal donors exhibited the average IL-7 level of 8 pg/ml with the maximum of 16.6 pg/ml of plasma. These results show that IL-7 production is not modified by SS anemia.

TGF-β Levels in LFSS, HFSS Patients and Control

Thirty-two SS patient and seven controls were evaluated for TGF-β plasma levels. The results depicted in Figure 6A showed that TGF-β plasma concentration was increased in SS anemia when compared with controls; however, a larger increase of TGF-β was detected in HFSS patients (94.9 ± 43 ng/ml n = 13) than in LFSS patients (54.1 ± 19.5 ng/ml n = 18). When a *t*-test assuming unequal variances is applied, the increase in TGF-β levels in HFSS patients is significant when compared with the controls ($P < 0.001$) and LFSS individuals ($P < 0.0044$), whereas the increase in TGF-β levels in LFSS patients is not significantly different from controls ($P < 0.132$).

In six SS patients, TGF-β was evaluated at several time points. As depicted in Figure 6B, despite some variation, TGF-β levels remained elevated in HFSS patients whereas the LFSS TGF-β values oscillated in the control range during follow-up.

LIF and MIP-1α Levels in SS Disease

The plasma levels of LIF and MIP-1α were also determined using an Elisa assay. None of the 32 plasmas tested for LIF were positive; six plasmas were positive for MIP-1α (46.9 up to 1,500 pg/ml), but no correlation with HbF levels was observed (data not shown).

DISCUSSION

The anemia of sickle cell disease is a complex phenomenon. Hemolysis is only one of the factors leading to the steady state level of Hb in this disease. Among nonhemolytic factors is the right shift of the oxygen equilibrium curve, which by delivering more oxygen per g of Hb than a Hb with a normal equilibrium curve, blunts the Epo response. In effect, there is no Epo elevation from normal values until the patient reaches about 9 g% of Hb [44]. Additionally, the blunted response below this threshold and lower level of Epo in adults compared with SS children [44], suggests that reduction in Epo secretion, possible secondary to silent renal damage, could be

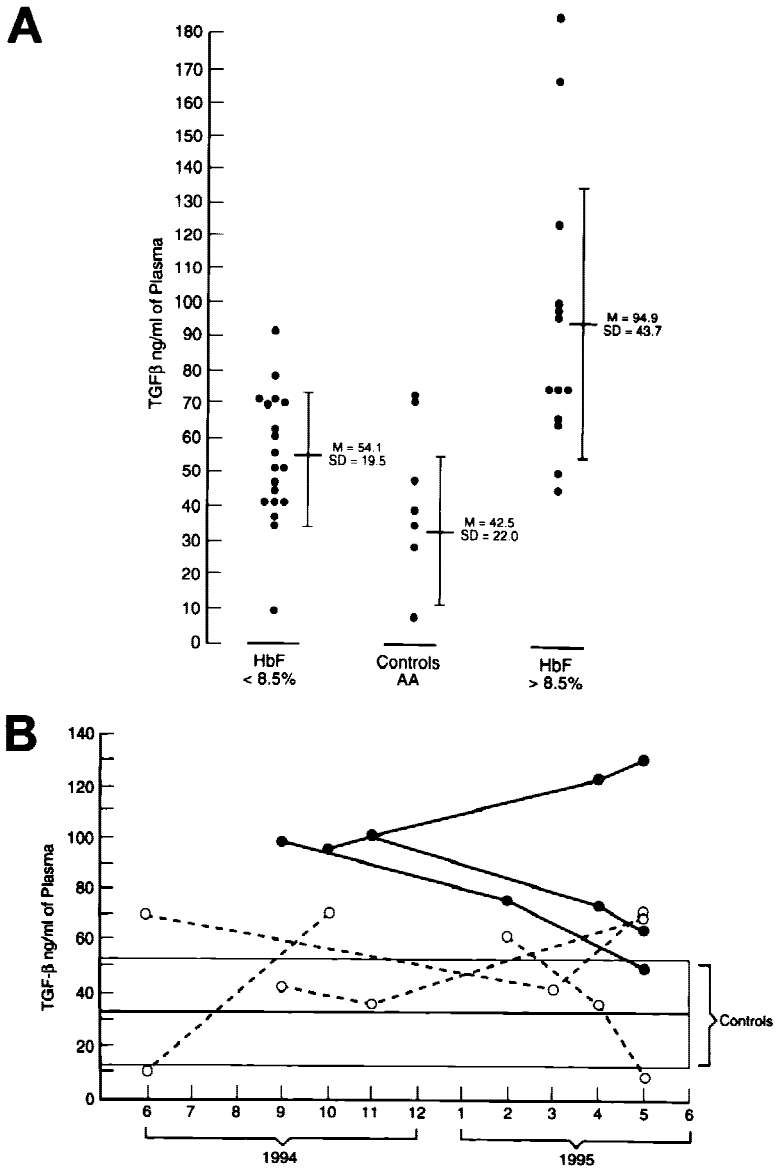

Fig. 6. A: TGF-β plasma levels were determined in 32 SS patients and 7 controls using an Elisa assay; the dots represent individual values. The patients were divided into two groups according to HbF levels (HbF < 8.5%; HbF > 8.5%). The mean values of TGF-β concentration in the group with low HbF was 54.1 ng/ml i.e. significantly lower ($p < 0.0044$) than in the group with high HbF (94.9 ng/ml). The mean value of TGF-β level in controls was 42.5 ng/ml. B: The TGF-β plasma levels were determined in three LFSS (○-open circle) and three HFSS (●-filled circle) patients three over a 1-year period. The values for each individual are related. The open box represents the mean and standard deviation of TGF-β plasma concentration in controls. The Figure illustrates that TGF-β remains elevated in HFSS patients during the follow-up whereas the LFSS TGF-β values oscillate in the control range.

involved. In any case, the anemia that stimulates erythropoiesis is activated below 9 g/dl of Hb.

We have recently reported [4] that sickle cell anemia is associated with increased mobilization of all myeloid stem cells that in some cases include LTC-IC. Furthermore, we demonstrated that more severe forms of SS disease (LFSS patients) exhibit increased levels of plasma GM-CSF, but not IL-3, and their monocyte conditioned medium lacks burst forming units-erythroid (BFU-E) inhibitory activity. In addition, LFSS circulating BFU-E are increased in number and exhibit active cycling as do LTC-IC; however, their numbers, in contrast to BFU-E, are in the normal range or decreased. This pattern suggests that, in these patients, increased LTC-IC proliferation is associated with a high rate of differentiation. On the other hand, intermediate forms of anemia (HFSS patients) are characterized by high levels of plasma IL-3, monocyte-derived inhibitory activity (or activities), and circulating BFU-E, as well as LTC-IC, are quiescent [43]. We hypothesize that in HFSS patients, a balance between inhibitory and stimulatory factors allows the appropriate proliferative stem cell response to a moderately increased demand. In contrast, lower levels of inhibitors, associated with active release of specific stimulatory factors, maintain a permanent state of overall increased hematopoiesis in LFSS patients, in response to continuous and severe red cell destruction.

We also present data based on sTfR plasma levels, which reinforces this model and further show that, while there is an overall increase of hematopoiesis in SS anemia, patients with low levels of HbF exhibit higher rates of erythropoiesis, as detected by high concentrations of sTfR, than individuals with increased levels of HbF. Since most TfRs are on erythroid progenitor cells, mea-

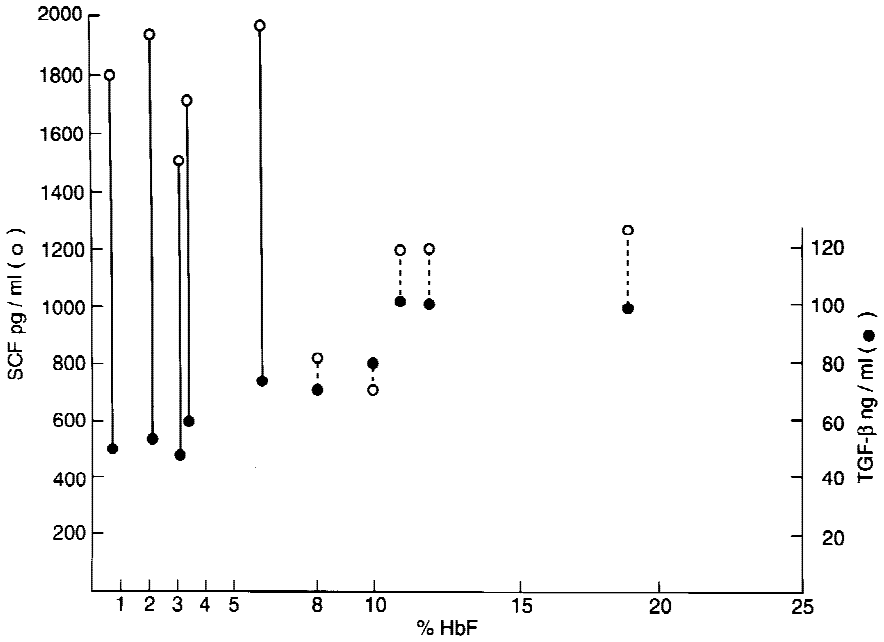

Fig. 7. SCF and TGF-β levels determined on the same samples. The open dots represent SCF individual values (pg/ml of plasma); the filled dots represent TGF-β values (ng/ml). The SCF TGF-β from each individual are related; continuous lines represent SS patients with low HbF% and high SCF levels; dashed lines are patients with low SCF and high HbF% and elevated TGF-β. The abscissa corresponds to the peripheral HbF concentration.

surement of plasma sTfR has been established as a sensitive indicator of the size of the erythroid pool, i.e., rate of erythropoiesis [11,12].

The fact that Epo concentrations were found to be higher in LFSS individuals further supports the idea that these patients experience a higher rate of erythropoiesis than HFSS subjects. The lack of good correlation between Epo levels and HbF is not contradictory with the above statement. As we mentioned before, the relation of Epo with anemia is complex, but in addition renal damage, chronic infection, and lack of a folic acid supplement may lead to inappropriate release of Epo and may explain the lack of correlation with HbF levels [44].

Our present data further suggests that the rate of hematopoiesis in SS disease is under control of a specific growth factor, i.e., SCF, and TGF-β, besides the previously reported GM-CSF and IL-3 [1–3]. We identified SCF as another *positive* regulator specifically affected by the presence of sickle cell anemia and its variable levels of severity. We demonstrated that TGF-β levels are differentially modified in HFSS patients when compared with LFSS patients and controls and that this pattern remains stable over time (one year). The levels of these two factors tend to exhibit a mirror image as a function of peripheral HbF (Fig. 7).

Furthermore, while increased G-CSF and IL-7 levels are occasionally observed in sickle cell anemia, they are probably not specific to SS disease because no relation to peripheral HbF concentration could be demonstrated. The same could be said of changes in LIF and MIP-1α.

What is the role of SCF in SS anemia? Based on the particular characteristics of LFSS patients, namely their higher level of circulating SCF and the larger proportion of circulating CD^{34} SCF^{+} cells detected in LFSS patients, we propose that SCF may be implicated in the regulation of the following events in the SS stem cells compartment:

1. *Regulation of the stem cells proliferation, in particular the early stem cells populations.* In favor of this hypothesis is the fact that LTC-ICs and BFU-Es from LFSS patients are actively cycling [1,43]. So it is possible that SCF, in a synergy with other factors, is required for recruitment/proliferation of LTC-IC and continuous BFU-E amplification, to ensure adequate production of the end red cells. This suggestion finds support in the observation that SCF is required for acute hematopoietic expansion during recovery from hemolytic anemia in adult mice [45]. Also, SCF in liquid cultures induces primitive stem cells [46–49] to generate a large number of colony-forming cells allowing the expansion of hematopoietic stem cells compartment. Moreover, SCF in a synergy with other cytokines, enhances the proliferative capacity of primitive hematopoietic progenitors and may accelerate the entry of hematopoietic stem cells into the cell cycle [17,50–54]. Finally, in combination with the other cytokines (in particular IL-6 and IL-6 receptors), SCF can also support the proliferation, differentiation, and terminal maturation of BFU-Es in the absence or low concentration of Epo [44,51] which may be particularly relevant to LFSS individuals with impaired Epo release [44].
2. *Regulation of the mobilization of stem cells from bone marrow to the circulation.* This hypothesis is supported by the report of Fleming et al. [55], that exogenous SCF treatment leads to the redistribution of SCF^{+} stem cells (Thy^{10}, Lin^{-10}, Sca^{-11}). Primates

treated with SCF exhibit a 10- to 100-fold increase in the number of circulating progenitor cells and mobilization of cells that engraft lethally irradiated recipients [56,57].

We have not found evidence to support the hypothesis proposed by Peschle et al. [58] that SCF can modulate fetal Hb synthesis in serum free cultures. In fact, the high performance liquid chromatography (HPLC) evaluation of HbF in BFU-E colonies from LFSS patients (data not shown) does not support this hypothesis.

The SCF level in HFSS patients is normal or lower than in controls, as expected according to our hypothesis that these individuals experienced moderate to low levels of anemia and do not require significantly elevated hematopoiesis. As will be discussed below, TGF-β may regulate SCF production [32] in these patients.

The hypothesis that SCF, with the help of other growth factors, plays an active role in SS disease, may appear to be in contradiction with data from aplastic anemia [59], myelodysplastic syndrome (with anemia) [60], and transplantation studies, that suggest that SCF serum levels are not clinically relevant to in vivo hematopoiesis, because they are not increased in these pathological conditions. Nevertheless, the pathophysiologies of the above diseases are very different from SS anemia (anemia from decreased production vs. anemia from increased destruction), including severe marrow microenvironment alterations not present in hemoglobinopathies. Hence, it is not surprising that different compensatory mechanisms are involved in these two conditions. Taken together, the data presented here are compatible with a model in which SCF may trigger the early stem cell proliferation/differentiation to CFC, which ultimately leads to the amplification of stem cells compartment, a situation much needed in LFSS individuals.

What is the role of TGF-β in SS anemia? We propose that the balance between the *negative* regulator, TGF-β, and the *positive* regulators SCF, IL-3, and GM-CSF, plays a major role in the control of hematopoiesis in SS disease. This statement is based on the data presented here and may be summarized as follows: 1. The patterns of plasma levels of TGF-β are different and characteristics in HFSS and LFSS patents; 2. High levels of TGF-β in HFSS patients are associated with lower levels of SCF suggesting that TGF-β may inhibit SCF production and/or repress hemopoietic progenitor expression of c-kit as demonstrated by a lower number of SCF^+ cells; 3. The slight increases of TGF-β in LFSS patients when compared with controls suggests that TGF-β in a synergy with GM-CSF may participate in amplification of GM-CSF responsive BFU-E population, according to the observation that TGF-β enhances growth of GM-CSF induced Lin^- cells [32].

Finally, G-CSF, IL-7, LIF, and MIP-1α do not seem to play a specific role in SS disease. We contend that the occasional elevation of G-CSF, IL-7, and MIP-1α is not part of SS pathology per se, but rather reflects a response to a nonspecific event (i.e., infection), because we did not find any correlation between these factors and HbF levels. However, we cannot exclude that LIF may be involved in stem cell control in situ in the SS bone marrow microenvironment; but, if so, the production and release of LIF are not increased in SS disease to such an extent to be detectable in plasma.

In conclusion, sickle cell anemia is characterized by various levels of hemopoietic stress linked to the levels of HbF as documented by the negative correlation of HbF with sTfR plasma concentrations. Hematopoietic regulation in LFSS and HFSS patients is characterized by different and specific patterns of SCF and TGF-β circulating levels, in addition to previously reported IL-3 and GM-CSF.

Furthermore, the data clearly indicate that SS disease is associated with specific disregulation of *early*-acting cytokines and not of later-acting lineage specific cytokines such as G-CSF. The occasional increase of the lineage specific factors, except Epo, reflects nonspecific SS disease events. Thus, in LFSS patients with a more severe form of disease, SCF with GM-CSF maintains a permanent response of early and late stem cells to increased needs, while recruitment of IL-3 dependent populations in moderate forms of anemia may be sufficient to maintain adequate levels of mature blood elements, and activation of SCF responsive early progenitors is not, or only occasionally required and is under negative control of TGF-β. Thus, the hemopoietic regulation of the target population in these patients may result from the positive action of IL-3 and eventually SCF and the negative action of TGF-β.

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